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Alterations of kynurenic acid content in the retina in response to retinal ganglion cell damage

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Abstract

The present study is the first to examine the modulation of retinal kynurenic acid (KYNA) content in response to *N*-methyl-D-aspartate (NMDA)-induced cell death in adult rat retinal ganglion cells (RGC). Adult Brown Norway rats were intravitreally injected with NMDA or PBS. Surviving RGC were retrogradely labeled with fluorogold and counted in wholemounts of retinas 2, 7 and 14 days after injection. Retinal KYNA content was measured by HPLC at the same time points. RGC numbers decreased significantly 2, 7 and 14 days after NMDA injection if compared to control retinas. KYNA concentration increased significantly two days after NMDA-injection. However, 7 and 14 days after injection retinal KYNA content was found markedly decreased in NMDA-treated eyes as compared to controls. It is conceivable that KYNA deficiency is causally related to the pathology of excitotoxic retinal diseases.

Keywords: KYNA; Retinal ganglion cell; NMDA; Rat; Neuroprotection

1. Introduction

The tryptophan metabolite kynurenic acid (KYNA) is the only known endogenous antagonist of ionotropic glutamate receptors and it acts as a neuroprotectant (Foster, Vezzani, French, & Schwarcz, 1984; Taber, Baker, & Fibiger, 1996).

In mammalian brain, KYNA is formed by irreversible transamination of L-kynurenine (L-KYN) by kynurenine aminotransferases (KAT I and KAT II) (Guidetti, Okuno, & Schwarcz, 1997). KAT I, a soluble enzyme which prefers pyruvate as a co-substrate (Okuno et al., 1990), has been localized immunohistochemically in the rat brain, medulla and spinal cord (Du et al., 1992; Kapoor, Okuno, Kido, & Kapoor, 1997; Knyihar-Csillik, Okuno, & Vecsei, 1999; Roberts, Du, McCarthy, Okuno, & Schwarcz, 1992). KAT II was first identified by Northern Blot *m*RNA analysis in the human brain (Okuno, Nakamura, & Schwarcz, 1991). KAT II, which in contrast to KAT I has a pH optimum in the physiological range (Okuno et al., 1991), appears to be responsible for most KYNA formation under physiologic conditions (Guidetti et al., 1997).

KYNA has been identified and quantified in the retina of adult rats (Rejdak et al., 2001). Retinal KYNA concentrations were in the same range as in the rabbit vitreous body (Zarnowski et al., 2001) and similar to those observed in the rat, rabbit and human brain (Moroni, Russi, Lombardi, Beni, & Carla, 1988; Turski et al., 1988). Immunohistochemical experiments using KAT I and KAT II antibodies (Okuno et al., 1991; Okuno, Tsujimoto, Nakamura, & Kido, 1993) showed that both enzymes are present in the rat inner retina. KAT I is preferentially expressed in Müller cell endfeet,

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while KAT II is localised in cells within the ganglion cell layer (Rejdak et al., 2001). Only recently, the presence of KYNA and developmental changes of its content in both vascularised rat and avascular chicken retinas during ontogeny have been reported (Rejdak et al., 2002).

The mammalian retina is highly susceptible to *N*-methyl-D-aspartate (NMDA) toxicity (Sabel, Sautter, Stoehr, & Siliprandi, 1995; Siliprandi et al., 1992), and NMDA-induced neuronal degeneration was found to be mainly restricted to the innermost retinal layers (Siliprandi et al., 1992). This pattern closely resembles retinal ganglion cells (RGC) loss in several ophthalmic diseases, including retinal ischemia, glaucoma and optic neuropathy (Dreyer, Pan, Storm, & Lipton, 1994; Sucher, Lipton, & Dreyer, 1997). If NMDA triggers calcium channels to induce apoptosis in neural tissue (Lam, Abler, Kwong, & Tso, 1999; Yu, Yeh, Strasser, Tian, & Choi, 1999) how does a spike in NMDA levels alter KYNA content? To answer this question the present study examined the effect of NMDA-induced RGC death on retinal KYNA content.

2. Material and methods

All experiments were performed in compliance with the guidelines of animal care in the European Community and the Association for Research in Vision and Ophthalmology.

2.1. Intravitreal injections of NMDA and PBS

Adult male Brown Norway rats were anaesthetized with an intraperitoneal injection of chloral hydrate (6 ml/kg body weight of a 7% solution). NMDA was dissolved in 0.2 M sterile phosphate-buffered saline (PBS, pH 7.4) at a final concentration of 10 mM for intravitreal injection. Eyes were injected intravitreally using a heat-pulled glass-capillary connected to a microsyringe (Drummond Scientific Co., Broomall, PA) under direct observation through the microscope. Animals with visible lens damage were excluded from the experiments and not analyzed thereafter.

A single injection of 2 μl of 10 mM NMDA (or 20 nmol) was given. Contralateral eyes served as control eyes and were injected with PBS.

2.2. Quantification of retinal ganglion cells

Labeling was performed either on the day of NMDA injection (for two-day survival) or five days after NMDA injection. Animals were anaesthetized deeply and a total of 7 μl of the fluorescent tracer hydroxystilbamidine methanesulfonate (Fluorogold, Molecular Probes, Eugene, OR) was applied into each of the superior colliculi by three stereotaxic injections. Two days later animals were killed by chloral hydrate overdose, the eyes were enucleated, retinas dissected, flat-mounted on cellulose nitrate filters (pore size 60 μm; Sartorius, Long Island, NY), and fixed in 2% PFA for 30 min. Labeled cells were defined as surviving. Observation was performed under a fluorescence microscope immediately, and counting was carried out in 12 distinct areas of 62,500 μm^2 per retina. Three areas per retinal quadrant at three different eccentricities of one-sixth, one-half, and five-sixths of the retinal radius were counted (Klöcker, Cellerino, & Bähr, 1998).

Images were obtained via a digital imaging system connected to the microscope (ImagePro), coded and analyzed semi-automatically in a masked fashion using a computer-assisted image analysis system.

2.3. Analysis of KYNA

Retinal KYNA content was measured 2, 7 and 14 days after NMDA injections. The animals were killed with CO_2, and the eyes were removed. To obtain isolated neural retinas, the eyes were opened along the ora serrata and cornea, and the lens and vitreous body were removed. Using a pair of forceps, the whole neural retinas were then carefully dissected free from the retinal pigment epithelium and sclera. Retinas were immediately frozen in liquid nitrogen after removal.

2.4. HPLC

KYNA levels were investigated according to the method of Turski and colleagues (Turski et al., 1988). Specimens were sonicated in 2 vols (w/v) of distilled water, immersed in a boiling water bath for 10 min and centrifuged (10 min, 20,000 rpm). The resulting supernatant was diluted (1:1) with 0.2 N HCl and applied to a Dowex 50-W hydrogen form pre-washed with 0.1 N HCl. Columns were subsequently washed with 1 ml 0.1 N HCl and 1 ml water. KYNA was eluted with 2 ml of water. The elute was subjected to HPLC and KYNA was detected fluorimetrically according to the method of Shibata (Shibata, 1988). HPLC reagents used in the study were obtained from Baker (Griesheim, Germany) and were of the highest available purity. Statistical analysis was performed using paired Student's *t* test.

3. Results

After injury (Mittag et al., 2000; Vorwerk et al., 1996b), RGC were labeled by stereotaxic injections into each superior colliculus. RGC numbers decreased significantly ($p < 0.001$) 2, 7 and 14 days after NMDA injection compared to control retinas. After two days, treatment with NMDA resulted in an RGC count of

1277 ± 68 (mean $\pm$ standard deviation) per mm^2 ($n = 4$), (PBS injected eyes showed ganglion cell counts of 2378 ± 81; $n = 4$). 557 ± 250 RGC/mm^2 survived seven days after NMDA injection ($n = 6$), (PBS injected eyes had 2329 ± 351 RGC/mm^2, $n = 7$). 14 days after NMDA injection, 224 ± 94 cells/mm^2 were counted ($n = 5$), compared to 2108 ± 189 RGC/mm^2 after PBS, $n = 5$ (Figs. 1 and 2). PBS injections did not have any influence on RGC survival if compared to untreated eyes (data not shown). The concentrations of KYNA measured in retinas of PBS injected eyes were in close agreement with those observed in the previous study (Rejdak et al., 2001).

NMDA-treated eyes displayed a significantly higher ($p = 0.03$) retinal KYNA concentration (136 ± 11 pmol/g wet wt, mean $\pm$ standard error, $n = 10$) two days after injection compared to PBS-injected group (109 ± 6 pmol/g wet wt, $n = 10$). However, seven days after injection retinal KYNA content markedly decreased ($p = 0.02$) to 83 ± 5 pmol/g wet wt. ($n = 9$) in NMDA-treated eyes compared to control values (118 ± 10 pmol/g wet wt, $n = 10$). A similar effect of NMDA-induced damage on retinal KYNA content was also observed 14 days after NMDA injection as compared to PBS-injected group (127.8 ± 9 pmol/g wet wt, $n = 7$) (91.6 ± 8 pmol/g wet wt, $n = 7$) (Fig. 3).

4. Discussion

The present study was designed to investigate the modulation of retinal KYNA content in response to NMDA toxicity to RGC at various time points following intravitreal NMDA injection. The loss of RGC is a hallmark of many ophthalmic diseases including glaucoma, retinal ischemia and optic neuropathy (Dreyer et al., 1994). Recent studies have indicated that glutamate affects RGC predominantly through the NMDA receptors (Sucher et al., 1997). It has been also suggested that AMPA and kainate receptor subtypes may also contribute to RGC loss (Otori, Wie, & Barnstable, 1998; Schuettauf, Naskar, Vorwerk, Zurakowski, & Dreyer, 2000). Since dysfunction of KYNA synthesis in the brain may be an important factor contributing to neuronal degeneration (Foster et al., 1984; Schwarcz et al., 1992), KYNA may have also relevance to the mechanisms of RGC loss.

Our study showed that RGC numbers decreased significantly two days after intraocular NMDA injections. During the same time, retinal KYNA contents increased markedly to 124% of control values. Increases in KYNA formation during the initial phases of excitotoxic injury may provide enhanced neuroprotection; it has been suggested that this is a factor in endogenous

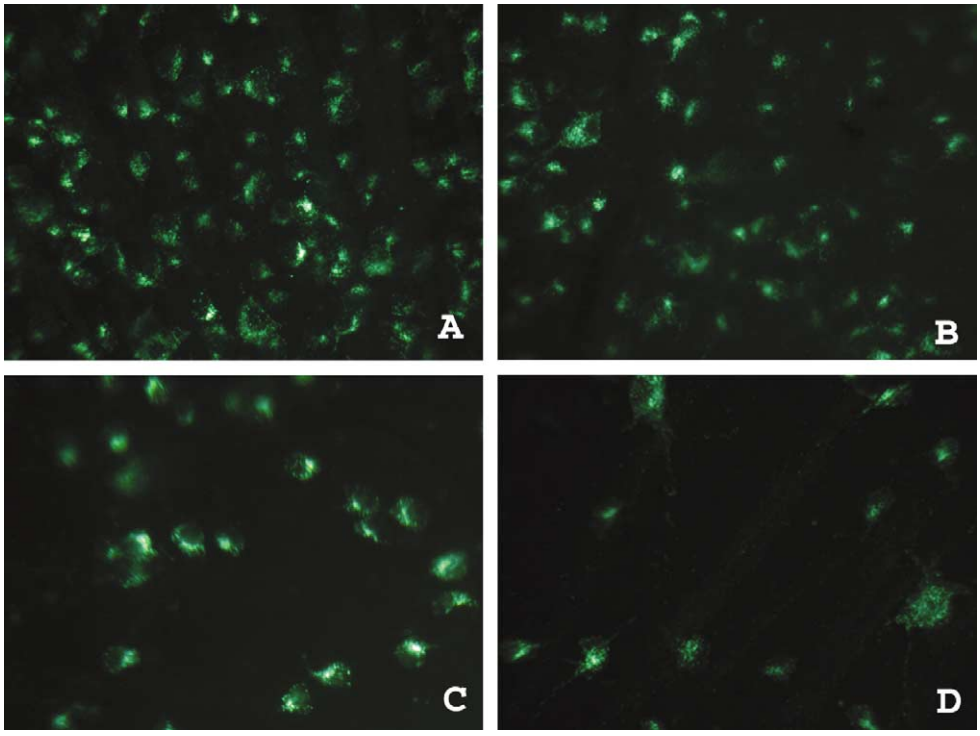

Fig. 1. Whole mounts of rat retina after NMDA injection; ganglion cells have been back-labeled by injection of fluorogold. (A) Control, (B) two days after injection, (C) seven days after injection, (D) 14 days after injection.

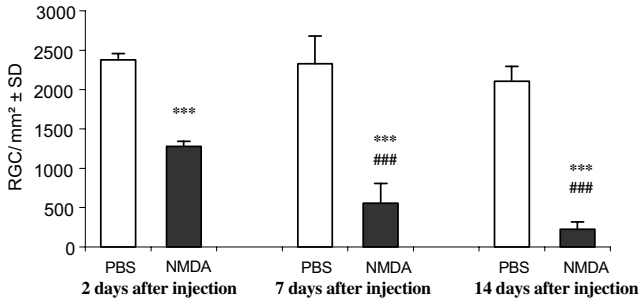

Fig. 2. RGC survival at 2, 7 and 14 days after NMDA or PBS injections. Values represent the mean ± standard deviation. (∗∗∗) indicates significantly higher RGC survival at all given time points compared to controls ($p < 0.001$ for each) by paired t-test. (###) indicates a significant loss of RGC between days 2 and 7 and days 7 and 14 after NMDA injection ($p < 0.001$ for each).

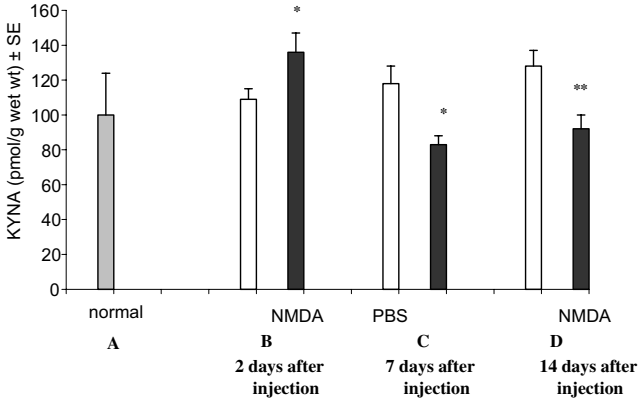

Fig. 3. Effect of NMDA-induced RGC damage on retinal KYNA content. Each column represents the mean KYNA concentration (pmol/g wt wet ± SE). White column—PBS-injected eyes, black column—NMDA-injected eyes. (A) Normal KYNA concentration in the retina (Rejdak et al., 2001), (B) two days after injection, (C) seven days after injection, (D) 14 days after injection. (∗) indicates significantly different KYNA content at all given time points if compared to controls ($p < 0.05$ for each) by paired t-test.

anti-excitotoxic defence mechanisms in the CNS (Urenjak & Obrenovitch, 2000). Such a lesion-induced rise in retinal KYNA levels may be explained either by an enhanced influx of blood-borne L-KYN due to a compromised blood-retina barrier (Hamilton & Gould, 1987) or by increased cerebral biosynthesis of L-KYN at the lesion site (Saito, Lackner, Markey, & Heyes, 1991). Other factors, like compounds released from damaged and dying neurons, activated microglia, and/or infiltrating macrophages may contribute as well (Marty, Dusart, & Peschanski, 1991). These compounds are known to play an active role during the initial days following excitotoxic insult (Lees, 1993). Interestingly, Ceresoli-Borroni et al., reported a dramatic increase of striatal KYNA content two days after quinolinate intrastriatal injections (Ceresoli-Borroni, Guidetti, & Schwarcz, 1999). Additionally, a similar effect was observed after an intrastriatal ibotenate injection (Ceresoli, Guidetti, & Schwarcz, 1997).

In our study, the retinas of NMDA-treated eyes displayed a marked decrease in RGC numbers 7 and 14 days after injection compared both to controls and to numbers observed two days after NMDA injection. Correspondingly, KYNA concentrations decreased to 70% of control values at days 7 and 14. It appears that the loss of RGC in that period of time might account for a decrease in retinal KYNA content, since immunohistochemical studies showed preferential localisation of KAT II on RGC (Rejdak et al., 2001). In contrast to the retina, KYNA content increased in the brain seven days after intrastriatal or intrahippocampal application of NMDA–agonist–quinolinate (Ceresoli-Borroni et al., 1999; Wu, Baran, Ungerstedt, & Schwarcz, 1992). This increase has been attributed to massive gliosis following neuronal cell death induced by neurotoxin (Ceresoli-Borroni et al., 1999; Wu et al., 1992). In the brain, KAT was found mainly in glia cells and it appears that they are responsible for most KYNA formation (Roberts et al., 1992; Tamburin, Mostardini, & Benatti, 1999). In the retina, neuronal damage due to neurotoxin administration induces reactive proliferation of Müller cells (Dyer & Cepko, 2000). It can be speculated, however, that these cells produce only limited amount of KYNA since they contain almost exclusively KAT I (Rejdak et al., 2001), which has an optimum at non-physiological pH of 9.5. This may explain the decrease found in our study in KYNA concentrations in retinas lesioned with NMDA.

KYNA deficiency has been suggested to be causally related to the pathology of excitotoxic brain diseases (Schwarcz et al., 1992). Decreased KYNA production was demonstrated in the brain of patients with Huntington's disease (Beal, Matson, Swartz, Gamache, & Bird, 1990; Jauch et al., 1995). Additionally, in vivo studies have shown that reduction of KYNA synthesis in the rat brain using non-specific inhibitors can lead to neurotoxicity (Beal, Ferrante, Swartz, & Kowall, 1991; Urbanska, Ikonomidou, Sieklucka, & Turski, 1991). Preferential loss of layer III of the entorhial cortex after local injection of a non-specific inhibitor of KYNA synthesis was suggested as a significant factor in the pathophysiology of temporal lobe epilepsy (Du, Eid, & Schwarcz, 1998), and is likely to be involved in the pathophysiology of several neuropsychiatric diseases, such as Alzheimer's disease and schizophrenia (Arnold, Hyman, Van Hoesen, & Damasio, 1991; Hyman, Kromer, & Van Hoesen, 1987). The decrease of KYNA after day 2 may have led to the dramatic loss of RGC seen at this timepoint in our experiments.

Otherwise, the endogenous glutamate antagonist KYNA was shown to prevent excitotoxic neuronal damage (Andine et al., 1988; Foster et al., 1984). It was found that an increase in brain KYNA concentration

either directly, or through enhanced KYNA synthesis may be neuroprotective. In experimental brain ischemia neuroprotection was observed following systemic administration of KYNA (Andine et al., 1988; Salvati et al., 1999), L-KYN (Nozaki & Beal, 1992) as well as kynurenine-3-hydroxylase inhibitors (Cozzi, Carpenedo, & Moroni, 1999; Moroni, Cozzi, Peruginelli, Carpenedo, & Pellegrini-Giampietro, 1999). Importantly, systemic L-KYN administration partially protects against NMDA-induced degeneration of RGC, and reduces visual discrimination deficits in adult rats (Vorwerk, Kreutz, Dreyer, & Sabel, 1996a).

Neuroprotective properties of KYNA are usually explained by its ability to block the excitatory amino acid receptor functions, but this is still debated. Under physiological conditions, KYNA concentrations (150 pmol/g in the human neocortex or at less than 50 pmol/g in the whole rat brain) are far lower than those required to antagonize EAA receptor functions established in in vitro electrophysiological studies ($IC_{50} = 8–15$ μM) (see Erhardt, Oberg, Mathe, & Engberg, 2001 and references cited there). Indeed, systemic administration of KYNA which produced neuroprotective effects in gerbils, was associated with a more than 250-fold transient rise of brain KYNA (Salvati et al., 1999). Regardless of its action on EAA receptors, KYNA is also a potent non-competitive antagonist of acetylcholine α7 nicotinic receptors, and it may also inhibit glutamate release indirectly via this mechanism (Carpenedo et al., 2001; Hilmas et al., 2001). A fourfold elevation of brain KYNA (to approx 125 pmol/g) by i.v. treatment of rats with PNU 156561A, a potent inhibitor of kynurenine 3-hydroxylase, was sufficient to abolish the glutamatergic component of activation of nigral dopaminergic neurons by nicotine (Erhardt et al., 2001). Thus, even a moderate increase in brain KYNA level may produce significant effects on the tonic glutamatergic control. In the retina, were able to show a moderate increase of KYNA two days after NMDA-induced RGC damage.

KYNA is formed by irreversible transamination of L-kynurenine (L-KYN) which is also a precursor of quinolinic acid (QUIN), 3OH-kynurenine (3OH-KYN) and 3OH-anthranilic acid. QUIN is an agonist of the subpopulation of NMDA receptors and neurotoxin. 3OH-kynurenine (3OH-KYN) and 3OH-anthranilic acid may cause both apoptotic and necrotic neuronal death (Moroni et al., 1999; Stone, 2001; Eastman & Guilarte, 1989). It has been found that pharmacological depletion of QUIN and 3OH-KYN synthesis exerts a protective effect in experimental brain ischemia (Moroni et al., 1999). Similar protective effects were observed after the enhancement of endogenous KYNA levels in the brain (Nozaki & Beal, 1992), indicating that an imbalance in kynurenine metabolism may have profound implications for neuronal function and cell loss, especially if that imbalance is present chronically (Stone, 1993).

In conclusion, retinal KYNA concentrations change in response to NMDA-induced RGC damage. The data demonstrate that there is a neuroprotective response to either toxic NMDA insult or RGC death. That response is an increase of KYNA. It is therefore conceivable that relative KYNA deficiency is an important factor contributing to the mechanisms of neuronal degeneration in the retina. Studies on KYNA synthesis in the retina in other model systems of retinal degeneration are recommended.

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